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(54) Title: IMMUNOTHERAPY

(57) Abstract: A method is provided for enhancing the reactivity of a T cell toward a tumour cell which method comprises: (a) isolating a T cell which is a tumour infiltrating lymphocyte (TIL) from a patient having said tumour cell present in their body; (b) introducing a nucleic acid sequence into the TIL, which sequence is capable of inhibiting or preventing expression of an endogenous Notch ligand in the TIL; and (c) re-introducing the transfected TIL into the patient; wherein the T cell comprises a T cell receptor specific for a tumour antigen expressed by the tumour cell.

IMMUNOTHERAPY

Field of the Invention

The present invention relates to methods for enhancing the response of T cells to tumour cells. These methods may be used in tumour immunotherapy.

Background to the Invention

Although significant advances have been made in recent years in the development of treatments for cancer, many of the resulting treatments are still based on administration of toxic compounds with consequential side-effects. As an alternative form of therapy, attempts have been made to enhance the patient's own immune response against tumours with varying degrees of success. It is not really understood how tumour cells escape recognition by the host immune system and/or inhibit an immune response. For example, it has been known for several years that a variety of immune cell-types infiltrate solid tumours in large numbers yet fail to mount an effective immunological response to the tumour. Indeed, it is possible to isolate tumour infiltrating lymphocytes which contain T cells with T cell receptors specific for tumour antigens *in vitro* which fail to be activated by tumour tissue *in vivo*. Thus there is need in the art for therapeutic methods that are capable of overcoming this immunosuppressive effect exerted by tumour cell *in vivo*.

Ligands that bind to cell surface receptors of the Notch family have recently been shown to be expressed on the surface of cells of the immune system, such as antigen presenting cells (APCs) and T-lymphocytes and a role for these molecules in such cells has been demonstrated in the regulation of tolerance induction (WO-A-98/20142).

It has recently been shown that it is possible to generate a class of regulatory T cells which are able to transmit antigen-specific tolerance to other T cells, a process termed infectious tolerance (WO-A-98/20142). The functional activity of these cells can be mimicked by over-expression of a Notch ligand protein on their cell surfaces. In particular, regulatory T cells can be generated by over-expression of a member of the Delta or Serrate family of Notch ligand proteins. Delta or Serrate expressing T cells specific to one antigenic epitope are also able to transfer tolerance to T cells recognising other epitopes on the same or related antigens, a phenomenon termed "epitope spreading".

Summary of the present invention

We have now found that the expression of Notch ligands is upregulated in some tumour cells. We have also shown that tumour cells that express a Notch ligand are capable of rendering T cells unresponsive to restimulation with specific antigen. Consequently, this may provide one possible means by which tumour cells may inhibit T cell responses. This conclusion is supported further by our previous results showing that upregulation of Notch ligand expression can induce immunotolerance (WO-A-98/20142). Thus, by preventing this mechanism operating in T cells *in vivo*, it may be possible to prevent tumour cells inducing immunotolerance in T cells that recognise tumour-specific antigens in the tumour cells. This would then allow the T cells to mount an immune response against the tumour cells.

Accordingly the present invention provides a method for enhancing the reactivity of a T cell toward a tumour cell which method comprises:

(a) isolating a T cell which preferably is a tumour infiltrating lymphocyte from a patient having said tumour cell present in their body;

(b) exposing the cell to an agent, which agent is capable of reducing or preventing expression of Notch or a Notch ligand in the cell; and

(c) re-introducing the cell into the patient;

wherein the T cell comprises a T cell receptor specific for a tumour antigen expressed by the tumour cell.

The present invention further provides a method for enhancing the reactivity of a T cell toward a tumour cell which method comprises:

(a) isolating an antigen presenting cell (APC) from a tumour present in the body of a patient;

(b) exposing the APC to an agent, which agent is capable of reducing or preventing expression of a Notch ligand in the T cell when the T cell is contacted with the transfected APC; and

(c) re-introducing the APC into the patient.

According to a further aspect, the present invention provides a method for enhancing the reactivity of a T cell toward a tumour cell which method comprises:

(a) isolating a tumour cell from a tumour present in the body of a patient;

(b) exposing the tumour cell to an agent, which is capable of reducing or preventing expression or interaction of an endogenous Notch or Notch ligand in the T cell when the T cell is contacted with the tumour cell; and

(c) re-introducing the tumour cell into the patient.

In one preferred embodiment step (b) comprises introducing a nucleic acid sequence into the cell, APC or tumour cell, which sequence is capable of reducing or preventing expression of Notch or a Notch ligand in the cell, APC or tumour cell.

Preferably the nucleic acid sequence encodes a polypeptide selected from Toll-like receptor protein family (Medzhitov *et al.*, 1997), a cytokine such as IL-12, IFN-γ, TNF-α, or a growth factor such as a bone morphogenetic protein (BMP), a

BMP receptor and activins. Preferably the polypeptide that decreases or interferes with the production of agents that are capable of producing an increase in the expression of Notch ligand, such as Noggin, Chordin, Follistatin, Xnr3, fibroblast growth factors and derivatives, fragments, variants and homologues thereof.

Alternatively, the nucleic acid sequence is an antisense construct derived from a sense nucleotide sequence encoding a polypeptide selected from a Notch ligand and a polypeptide capable of upregulating Notch ligand expression, such as Noggin, Chordin, Follistatin, Xnr3, fibroblast growth factors and derivatives, fragments, variants and homologues thereof.

In another preferred embodiment the agent is a chemical compound such as a polypeptide which is exposed/incubated with the cell, APC or tumour cell. The agent should be one which is capable of modulating Notch-Notch ligand interactions. In this embodiment the polypeptide is preferably selected from a Toll-like receptor, a cytokine such as IL-12, IFN-γ, TNF-α, or a growth factor such as a BMP, a BMP receptor and activins. Preferably the polypeptide decreases or interferes with the production of an agent that is capable of producing an increase in the expression of Notch ligand, such as Noggin, Chordin, Follistatin, Xnr3, fibroblast growth factors and derivatives, fragments, variants, homologues and analogs thereof.

Preferably when the agent is a receptor or a nucleic acid sequence encoding a receptor, the receptor is activated. Thus, e.g., when the agent is a nucleic acid sequence, the receptor is constitutively active when expressed.

As used herein, the terms protein and polypeptide may be assumed to be synonymous, protein merely being used in a general sense to indicate a relatively longer amino acid sequence than that present in a polypeptide.

The term "derivative" as used herein, in relation to proteins or polypeptides of the present invention includes any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acid residues from or to the sequence providing that the resultant protein or polypeptide possesses the capability of modulating Notch-Notch ligand interactions.

The term "variant" as used herein, in relation to proteins or polypeptides of the present invention includes any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acid residues from or to the sequence providing that the resultant protein or polypeptide possesses the capability of modulating Notch-Notch ligand interactions.

The term "analog" are used herein, in relation to the proteins or polypeptides of the present invention includes any peptidomimetic, that is, a chemical compound that possesses the capability of modulating Notch-Notch ligand interactions in a similar manner to the parent protein or polypeptide. These include compounds that may antagonise the expression or activity of a Notch-protein or Notch-ligand.

An agent may be considered to modulate Notch-Notch ligand interactions if it is capable of inhibiting the interaction of Notch with its ligands, preferably to an extent sufficient to provide therapeutic efficacy.

In a preferred embodiment the agent modulates Notch-Notch ligand interactions by being capable of reducing or preventing expression of Notch or Notch ligand.

The expression "Notch-Notch ligand" as used herein means the interaction between a Notch family member and a ligand capable of binding to one or more such member. Thus by the expression "reducing or preventing interaction of Notch or a Notch-ligand" we mean inhibiting the interaction of Notch in a T cell,

APC or tumour cell with a Notch ligand or inhibiting the interaction of a Notch ligand in a T cell, APC or tumour cell with Notch.

The term therapy are used herein should be taken to encompass diagnostic and prophylatic applications.

Preferably the endogenous Notch ligand is selected from Serrate, Delta and homologues thereof, more preferably Serrate and Delta.

The present invention also provides a method of vaccinating a patient against a tumour which method comprises:

(a) administering a tumour antigen expressed by the tumour to the skin of the patient; and

(b) exposing an agent to an APC present in the skin of the patient wherein the agent is capable of inhibiting or preventing expression of a Notch ligand in a T cell.

In particular, where the nucleic acid sequence is introduced into an APC, the sequence is preferably capable of inhibiting or reducing the expression of Serrate-1 since expression of Serrate-1 is down-regulated in APCs during an effective immune response and up-regulated in conditions of immune tolerance i.e. Serrate-1 is a mediator of tolerance induction. An example of a suitable sequence is therefore a sequence an activated Toll-like receptor (for example TLR-2, TLR-4) which would lead to decreased expression of Serrate-1, Serrate-2, Delta-1 and Delta-3.

Detailed Description of the Invention

A. Notch and Notch ligands

An endogenous Notch or Notch ligand in the context of the present invention is a polypeptide encoded by the genome of a mammalian cell that is capable of being

expressed by the mammalian cell. In particular the mammalian cell may be a hemapoietic cell such as a T-cell or an antigen presenting cell. The endogenous Notch ligand is typically is capable of binding to a Notch receptor polypeptide present in the membrane of a variety of mammalian cell types, for example hemapoietic stem cells. At least four Notch receptors (Notch-1, Notch-2, Notch-3 and Notch-4) have been identified to date in human cells.

Particular examples of mammalian Notch ligands identified to date include the Delta family, for example Delta (Genbank Accession No. AF003522 - *Homo sapiens*), Delta-3 (Genbank Accession No. AF084576 - *Rattus norvegicus*) and Delta-like 3 (*Mus musculus*), the Serrate family, for example Serrate-1 and Serrate-2 (WO97/01571, WO96/27610 and WO92/19734), Jagged-1 and Jagged-2 (Genbank Accession No. AF029778 - *Homo sapiens*), and LAG-2. Homology between family members is extensive. For example, human Jagged-2 has 40.6% identity and 58.7% similarity to Serrate.

Further homologues of known mammalian Notch ligands may be identified using standard techniques. By a "homologue" it is meant a gene product that exhibits sequence homology, either amino acid or nucleic acid sequence homology, to any one of the known Notch ligands, for example as mentioned above. Typically, a homologue of a known Notch ligand will be at least 20%, preferably at least 30%, identical at the amino acid level to the corresponding known Notch ligand. Techniques and software for calculating sequence homology between two or more amino acid or nucleic acid sequences are well known in the art (see for example http://www.ncbi.nlm.nih.gov and Ausubel *et al.*, Current Protocols in Molecular Biology (1995), John Wiley & Sons, Inc.)

Notch ligands identified to date have a diagnostic DSL domain (D. *Delta*, S. *Serrate*, L. Lag2) comprising 20 to 22 amino acids at the amino terminus of the protein and between 3 to 8 EGF-like repeats on the extracellular surface. It is

therefore preferred that homologues of Notch ligands also comprise a DSL domain at the N-terminus and between 3 to 8 EGF-like repeats on the extracellular surface.

In addition, suitable homologues will be capable of binding to a Notch receptor. Binding may be assessed by a variety of techniques known in the art including *in vitro* binding assays.

Homologues of Notch ligands can be identified in a number of ways, for example by probing genomic or cDNA libraries with probes comprising all or part of a nucleic acid encoding a Notch ligand under conditions of medium to high stringency (for example 0.03M sodium chloride and 0.03M sodium citrate at from about 50°C to about 60°C). Alternatively, homologues may also be obtained using degenerate PCR which will generally use primers designed to target sequences within the variants and homologues encoding conserved amino acid sequences. The primers will contain one or more degenerate positions and will be used at stringency conditions lower than those used for cloning sequences with single sequence primers against known sequences.

B. Nucleic acid sequences capable of reducing or preventing endogenous Notch ligand expression

Substances that may be used to inhibit Notch ligand expression include nucleic acid sequences encoding polypeptides that affect the expression of genes encoding Notch ligands. For instance, for Delta expression, binding of extracellular BMPs (bone morphogenetic proteins, Wilson and Hemmati-Brivanlou, 1997; Hemmati-Brivanlou and Melton, 1997) to their receptors leads to down-regulated Delta transcription due to the inhibition of the expression of transcription factors of the achaete/scute complex. This complex is believed to be directly involved in the regulation of Delta expression. Thus, any polypeptide that upregulates BMP expression and/or stimulates the binding of

BMPs to their receptors may be capable of producing a decrease in the expression of Notch ligands such as Delta and/or Serrate. Examples may include nucleic acids encoding BMPs themselves. Furthermore, any substance that inhibits expression of transcription factors of the achaete/scute complex may also downregulate Notch ligand expression.

Members of the BMP family include BMP1 to BMP6, BMP7 also called OP1, OP2 (BMP8) and others. BMPs belong to the transforming growth factor beta (TGF-beta) superfamily, which includes, in addition to the TGF-betas, activins/inhibins (e.g., alpha-inhibin), mullerian inhibiting substance, and glial cell line-derived neurotrophic factor.

Other examples of polypeptides that inhibit the expression of Delta and/or Serrate include the Toll-like receptor (Medzhitov *et al.*, 1997) or any other receptors linked to the innate immune system (for example CD14, complement receptors, scavenger receptors or defensin proteins), and other polypeptides that decrease or interfere with the production of Noggin (Valenzuela *et al.*, 1995), Chordin (Sasai *et al.*, 1994), Follistatin (Iemura *et al.*, 1998), Xnr3, and derivatives and variants thereof. Noggin and Chordin bind to BMPs thereby preventing activation of their signalling cascade which leads to decreased Delta transcription. Consequently, reducing Noggin and Chordin levels may lead to decrease Notch ligand, in particular Delta, expression.

In more detail, in Drosophila, the Toll transmembrane receptor plays a central role in the signalling pathways that control amongst other things the innate nonspecific immune response. This Toll-mediated immune response reflects an ancestral conserved signalling system that has homologous components in a wide range of organisms. Human Toll homologues have been identified amongst the Toll-like receptor (TLR) genes and Toll/interleukin-1 receptor-like (TIL) genes and contain the characteristic Toll motifs: an extracellular leucine-rich repeat

domain and a cytoplasmic interleukin-1 receptor-like region. The Toll-like receptor genes (including TIL genes) now include TLR4, TIL3, TIL4, and 4 other identified TLR genes.

Other suitable sequences that may be used to downregulate Notch ligand expression include Mesp2 (Takahashi *et al.*, 2000), those encoding immune costimulatory molecules (for example CD80, CD86, ICOS, SLAM) and other accessory molecules that are associated with immune potentiation (for example CD2, LFA-1).

Other suitable substances that may be used to downregulate Notch ligand expression include nucleic acids that inhibit the effect of transforming growth factors such as members of the fibroblast growth factor (FGF) family. The FGF may be a mammalian basic FGF, acidic FGF or another member of the FGF family such as an FGF-1, FGF-2, FGF-3, FGF-4, FGF-5, FGF-6, FGF-7. Preferably the FGF is not acidic FGF (FGF-1; Zhao *et al.*, 1995). Most preferably, the FGF is a member of the FGF family which acts by stimulating the upregulation of expression of a Serrate polypeptide on APCs. The inventors have shown that members of the FGF family can upregulate Serrate-1 gene expression in APCs.

Suitable nucleic acid sequences may include anti-sense constructs, for example nucleic acid sequences encoding antisense Notch ligand constructs as well as antisense constructs designed to reduce or inhibit the expression of upregulators of Notch ligand expression (see above). The antisense nucleic acid may be an oligonucleotide such as a synthetic single-stranded DNA. However, more preferably, the antisense is an antisense RNA produced in the patient's own cells as a result of introduction of a genetic vector. The vector is responsible for production of antisense RNA of the desired specificity on introduction of the vector into a host cell.

Preferably, the nucleic acid sequence for use in the present invention is capable of inhibiting Serrate and Delta, preferably Serrate 1 and Serrate 2 as well as Delta 1 and Delta 3 expression in APCs such as dendritic cells. In particular, the nucleic acid sequence may be capable of inhibiting Serrate expression but not Delta expression in APCs. Alternatively, the nucleic acid sequence for use in the present invention is capable of inhibiting Delta expression in T cells such as $CD4^+$ helper T cells or other cells of the immune system that express Delta (for example in response to stimulation of cell surface receptors). In particular, the nucleic acid sequence may be capable of inhibiting Delta expression but not Serrate expression in T cells. In a particularly preferred embodiment, the nucleic acid sequence is capable of inhibiting Notch ligand expression in both T cells and APC, for example Serrate expression in APCs and Delta expression in T cells.

Suitable nucleic acid sequences for use according to the present invention may be conveniently identified using a simple screening procedure. In one such assay procedure, T cells, APCs or tumour cells in culture may be transfected with a candidate sequence and the effect on expression of an endogenous Notch ligand, such as Delta or Serrate, determined in the presence or absence of a suitable stimulus (such as an antigen) for example by (i) measuring transcription initiated from the gene encoding the Notch ligand (see, for example WO-A-98/20142) or by quantitative-reverse transcriptase-polymerase chain reaction (RT-PCR)(see Example 1); (ii) detecting Notch ligand protein by techniques such as Western blotting of cell extracts, immunohistochemistry or flow cytometry; and/or (iii) functional assays such as cell adhesion assays.

C. Compounds capable of reducing or preventing Notch or Notch ligand expression or interaction

Substances that may be used to inhibit Notch and Notch ligand expression

polypeptides and conventional chemical compounds. The polypeptides may be the expression products of the nucleic acid sequences mentioned above in section B.

Preferred suitable substances that may be used to downregulate Notch ligand expression include growth factors and cytokines. More preferably soluble protein growth factors may be used to inhibit Notch or Notch ligand expression on APCs, T cells or tumour cells in culture by the addition of exogenous growth factors. For instance, Notch ligand expression may be reduced or inhibited by the addition of BMPs or activins (a member of the TGF-b superfamily). In addition, T cells, APCs or tumour cells could be cultured in the presence of inflammatory type cytokines including IL-12, IFN-γ, IL-18, TNF-α, either alone or in combination with BMPs.

The present invention also relates to modification of Notch-protein expression or presentation on the cell membrane or signalling pathways. Agents that reduce or interfere with its presentation as a fully functional cell membrane protein may include MMP inhibitors such as hydroxymate-based inhibitors.

Other substances which may be used to reduce interaction between Notch and Notch ligands are exogenous Notch or Notch ligands or functional derivatives thereof. Such Notch ligand derivatives would preferably have the DSL domain at the N-terminus and between 3 to 8 EGF-like repeats on the extracellular surface. A peptide corresponding to the Delta/Serrate/LAG-2 domain of hJagged1 and supernatants from COS cells expressing a soluble form of the extracellular portion of hJagged1 was found to mimic the effect of Jagged1 in inhibiting Notch1 (Li *et al.*, 1998). Other substances which may be used to reduce interaction between Notch and Notch ligands include, for example, Numb (Eddison *et al.*, 2000) and Scabrous (Lee *et al.*, 2000).

Whether a substance can be used for modulating Notch-Notch ligand expression may be determined using suitable screening assays.

Screening assays for the detection of decreased Notch, Notch ligand expression and/or processing include:

Notch-Notch ligand expression may be assessed following exposure of isolated cells to test compounds in culture using for example:

(a) at the protein level by specific antibody staining using immunohistochemistry or flow cytometry.

(b) at the RNA level by quantitative - reverse transcriptase-polymerase chain reaction (RT-PCR). RT-PCR may be performed using a control plasmid with in-built standards for measuring endogenous gene expression with primers specific for Notch 1 and Notch 2, Serrate 1 and Serrate 2, Delta 1 and Delta 2 and Delta 3. This construct may be modified as new ligand members are identified.

(c) at the functional level in cell adhesion assays.

Decreased Notch ligand or Notch expression should lead to decreased adhesion between cells expressing Notch and its ligands. Test cells will be exposed to a particular treatment in culture and radiolabelled or flourescein labelled target cells (transfected with Notch/Notch ligand protein) will be overlayed. Cell mixtures will be incubated at 37°C for 2 hours. Nonadherent cells will be washed away and the level of adherence measured by the level of radioactivity/immunofluorescence at the plate surface.

Using such methods it is possible to detect compounds or Notch-ligands that affect the expression or processing of a Notch-protein or Notch-ligand. The

invention also relates to compounds, or Notch-ligands detectable by these assays methods, and also to their use in the methods of the present invention.

D. Transgenic animals

The present invention also relates to cell lines or transgenic animals which are capable of expressing or overexpressing Notch, a Notch ligand or at least one agent useful in the present invention. Preferably the cell line or animal expresses or overexpresses Notch, Delta or Serrate.

The present invention additionally relates to cell lines or transgenic animals which are capable of expressing or overexpressing at least one polypeptide which is capable of inhibiting Notch-Notch ligand interactions. Such agents have been described above and for the avoidance of doubt are specifically incorporated herein by reference.

The present invention further relates to cell lines or transgenic animals which are capable of expressing or overexpressing at least one polypeptide which is capable of enhancing Notch-Notch ligand interactions. Agents that enhance the presentation of a fully functional Notch-protein on the T cell or APC surface include matrix metalloproteinases such as the product of the Kuzbanian gene of Drosophila (Dkuz *et al.*, (1997) and other ADAMALYSIN gene family members. Suitable agents that influence expression of Notch-ligands include agents that affect the expression of Delta and/or Serrate genes. For instance, for Delta expression, any agent that inhibits the binding of BMPs to their receptors is capable of producing an increase in the expression of Delta and/or Serrate. Such agents include Noggin, Chordin, Follistatin, FGFs, Fringe and derivatives and variants thereof.

The transgenic animal is typically a vertebrate, more preferably a rodent, such as

a rat or a mouse, but also includes other mammals such as human, goat, pig or cow etc.

Such transgenic animals are useful as animal models of disease and in screening assays for new useful compounds. By specifically expressing one or more polypeptides, as defined above, the effect of such polypeptides on the development of disease can be studied. Furthermore, therapies including gene therapy and various drugs can be tested on transgenic animals. Methods for the production of transgenic animals are known in the art. For example, there are several possible routes for the introduction of genes into embryos. These include (i) direct transfection or retroviral infection of embryonic stem cells followed by introduction of these cells into an embryo at the blastocyst stage of development; (ii) retroviral infection of early embryos; and (iii) direct microinjection of DNA into zygotes or early embryo cells.

The present invention also includes stable cell lines for use as disease models for testing or treatment. A stable cell line will contain a recombinant gene or genes, also known herein as a transgene.

A cell line containing a transgene, as described herein, is made by introducing the transgene into a selected cell line according to one of several procedures known in the art for introducing a foreign gene into a cell.

The sequences encoding the inhibitors and enhancers of Notch-Notch ligand interactions as well as Notch or a Notch ligand itself are operably linked to control sequences, including promoters/enhancers and other expression regulation signals.

The promoter is typically selected from promoters which are functional in mammalian cells, although prokaryotic promoters and promoters functional in

other eukaryotic cells may be used. The promoter is typically derived from promoter sequences of viral or eukaryotic genes. For example, it may be a promoter derived from the genome of a cell in which expression is to occur. With respect to eukaryotic promoters, they may be promoters that function in a ubiquitous manner (such as promoters of a-actin, b-actin, tubulin) or, alternatively, a tissue-specific manner (such as promoters of the genes for pyruvate kinase). Tissue-specific promoters specific for lymptocytes, dendritic cells, skin, brain cells and epithelial cells within the eye are particularly preferred, for example the CD2, CD11c, keratin 14, Wnt-1 and Rhodopsin promoters respectively. Preferably the epithelial cell promoter SPC is used. They may also be_promoters that respond to specific stimuli, for example promoters that bind steroid hormone receptors. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR) promoter, the rous sarcoma virus (RSV) LTR promoter or the human cytomegalovirus (CMV) IE promoter.

It may also be advantageous for the promoters to be inducible so that the levels of expression of the heterologous gene can be regulated during the life-time of the cell. Inducible means that the levels of expression obtained using the promoter can be regulated.

In addition, any of these promoters may be modified by the addition of further regulatory sequences, for example enhancer sequences. Chimeric promoters may also be used comprising sequence elements from two or more different promoters described above.

E. Tumour cells expressing Notch ligand

We have already identified expression of Notch ligands in melanoma cell lines. Other tumour cells may also be tested for expression of Notch ligands using a

variety of techniques known in the art such as detection of mRNA by RT-PCR or detection of the Notch ligand polypeptides by Western blotting. Suitable tumour cells to be tested include cells present in malignancies such as cancer of the breast, cervix, colon, rectum, endometrium, kidney, lung, ovary, pancreas, prostate gland, skin, stomach, bladder, CNS, oesophagus, head-or-neck, liver, testis, thymus or thyroid. Malignant blood cells, bone marrow cells, B-lymphocytes, T-lymphocytes, lymphocytic progenitors or myeloid cell progenitors may also be tested.

The tumour cell to be tested for Notch ligand expression may be a tumour cell from a solid tumour or a non-solid tumour and may be a primary tumour cell or a disseminated metastatic (secondary) tumour cell. Non-solid tumours include myeloma; leukaemia (acute or chronic, lymphocytic or myelocytic) such as acute myeloblastic, acute promyelocytic, acute myelomonocytic, acute monocytic, erythroleukaemia; and lymphomas such as Hodgkin's, non-Hodgkin's and Burkitt's. Solid tumours include carcinoma, colon carcinoma, small cell lung carcinoma, non-small cell lung carcinoma, adenocarcinoma, melanoma, basal or squamous cell carcinoma, mesothelioma, adenocarcinoma, neuroblastoma, glioma, astrocytoma, medulloblastoma, retinoblastoma, sarcoma, osteosarcoma, rhabdomyosarcoma, fibrosarcoma, osteogenic sarcoma, hepatoma, and seminoma.

F. Antigen Presenting Cells and T cells

Antigen-presenting cells (APCs) for use in the present invention may be "professional" antigen presenting cells or may be another cell that may be induced to present antigen to T cells. Alternatively a APC precursor may be used which differentiates or is activated under the conditions of culture to produce an APC. An APC for use in the *ex vivo* methods of the invention is typically isolated from a tumour or peripheral blood found within the body of a patient.

Preferably the APC or precursor is of human origin. However, where APCs are used in preliminary *in vitro* screening procedures to identify and test suitable nucleic acid sequences, APCs from any suitable source, such as a healthy patient, may be used.

APCs include dendritic cells (DCs) such as interdigitating DCs or follicular DCs, Langerhans cells, PBMCs, macrophages, B-lymphocytes, T-lymphocytes, or other cell types such as epithelial cells, fibroblasts or endothelial cells, activated or engineered by transfection to express a MHC molecule (Class I or II) on their surfaces. Precursors of APCs include $CD34^+$ cells, monocytes, fibroblasts and endothelial cells. The APCs or precursors may be modified by the culture conditions or may be genetically modified, for instance by transfection of one or more genes encoding proteins which play a role in antigen presentation and/or in combination of selected cytokine genes which would promote to immune potentiation (for example IL-2, IL-12, IFN-γ, TNF-α, IL-18 etc.). Such proteins include MHC molecules (Class I or Class II), CD80, CD86, or CD40. Most preferably DCs or DC-precursors are included as a source of APCs.

Dendritic cells (DCs) can be isolated/prepared by a number of means, for example they can either be purified directly from peripheral blood, or generated from $CD34^+$ precursor cells for example after mobilisation into peripheral blood by treatment with GM-CSF, or directly from bone marrow. From peripheral blood, adherent precursors can be treated with a GM-CSF/IL-4 mixture (Inaba *et al.*, 1992), or from bone marrow, non-adherent $CD34^+$ cells can be treated with GM-CSF and TNF-a (Caux *et al.*, 1992). DCs can also be routinely prepared from the peripheral blood of human volunteers, similarly to the method of Sallusto and Lanzavecchia (1994) using purified peripheral blood mononucleocytes (PBMCs) and treating 2 hour adherent cells with GM-CSF and IL-4. If required, these may be depleted of $CD19^+$ B cells and $CD3^+$, $CD2^+$ T cells using magnetic beads (see Coffin *et al.*, 1998). Culture conditions may include

other cytokines such as GM-CSF or IL-4 for the maintenance and, or activity of the dendritic cells or other antigen presenting cells.

Where T cells are to be used in the *ex vivo* methods of the invention, the T cells are typically infiltrating T lymphocytes isolated from a solid tumour within the body of an individual suffering from a cancer. However, where T cells are used in preliminary *in vitro* screening procedures to identify and test suitable nucleic acid sequences, T cells from any suitable source, such as a healthy patient, may be used and may be obtained from blood or another source (such as lymph nodes, spleen, or bone marrow). They may optionally be enriched or purified by standard procedures. The T cells may be used in combination with other immune cells, obtained from the same or a different individual. Alternatively whole blood may be used or leukocyte enriched blood or purified white blood cells as a source of T cells and other cell types. It is particularly preferred to use helper T cells ($CD4^+$). Alternatively other T cells such as $CD8^+$ cells may be used. It may also be convenient to use cell lines such as T cell hybridomas.

Lymphocytes with antigen receptors recognising antigens presented by tumour cells (tumour-reactive lymphocytes (TRLs)) can be isolated from peripheral blood, lymph nodes or from tumour tissue (tumour-infiltrating lymphocytes (TILs)). Methods for isolating and culturing TRLs are well known in the art. See for example Vose *et al.* (1977). TILs and other TRLs may be isolated and expanded in culture in the presence of cytokines such as Interleukin (IL)-2, IL-12, IFN-γ, TNF-α, IL-18 as described by Belldegrun *et al.* (1988); Belldegrun *et al.* (1989); and Spiess *et al.* (1987). TRLs and TILs reactive with identified tumour antigens can also be isolated using MHC Class-I and Class-II tetramer technology (Dunbar *et al.*, 1998; Romero *et al.*, 1998).

Thus, it will be understood that the term "antigen presenting cell or the like" are used herein is not intended to be limited to APCs. The skilled man will

understand that any vehicle capable of presenting to the T cell population may be used, for the sake of convenience the term APCs is used to refer to all these. As indicated above, preferred examples of suitable APCs include dendritic cells, L cells, hybridomas, fibroblasts, lymphomas, macrophages, B cells or synthetic APCs such as lipid membranes.

G. Introduction of nucleic acid sequences into APCs, T cells and Tumour cells

T cells/APCs/tumour cells as described above are cultured in a suitable culture medium such as DMEM or other defined media, optionally in the presence of fetal calf serum. If required, a small aliquot of cells may be tested for down regulation of Notch ligand expression as described above. Alternatively, cell activity may be measured by the inhibition of T-cell proliferation as described in WO98/20142. T-cells/APCs/tumour cells transfected with a nucleic acid construct directing the expression of, for example Serrate, may be used as a control.

As discussed above, polypeptide substances may be administered to T cells/APCs/tumour cells by introducing nucleic acid constructs/viral vectors encoding the polypeptide into cells under conditions that allow for expression of the polypeptide in the T cell/APC/tumour cell. Similarly, nucleic acid constructs encoding antisense constructs may be introduced into the T cells/APCs/tumour cells by transfection, viral infection or viral transduction.

The resulting T cells/APCs/tumour cell that comprise nucleic acid constructs capable of downregulating Notch ligand expression are now ready for use. For example, they may be prepared for administration to a patient or incubated with T cells *in vitro* (*ex vivo*).

H. Exposure of agent to APCs and T cells

T cells/APCs/tumour cells may be cultured as described above. The APCs/T cells/tumour cells may be incubated/exposed to substances which are capable of interferring with or downregulating Notch or Notch ligand expression. The resulting T cells/APCs/tumour cells that have downregulated Notch or Notch ligand expression are now ready for use. For example, they may be prepared for administration to a patient or incubated with T cells *in vitro* (*ex vivo*).

For example, tumour material may be isolated and transfected with a nucleic acid sequence which encodes for, e.g., a Toll-like receptor or BMP receptor and/or costimulatory molecules (suitable costimulants are mentioned above) and/or treated with cytokines, e.g. IFN-γ, TNF-α, IL-12, and then used *in vitro* to prime TRL and/or TIL cells.

I. Therapeutic Uses

The T cells/APCs/tumour cells prepared by the method of the invention may be administered to a patient suffering from a malignancy, the malignancy typically comprising cancerous cells that express a Notch ligand. The presence of cancerous cells that express, in particular over-express, a Notch ligand may be determined by, for example, testing using the methods described above a sample of cancerous tissue obtained from the patient.

Generally, the patient will be the same patient from whom the treated T cells/APCs/tumour cells originated. Examples of malignancies that may be treated include cancer of the breast, cervix, colon, rectum, endometrium, kidney, lung, ovary, pancreas, prostate gland, skin, stomach, bladder, CNS, oesophagus, head-or-neck, liver, testis, thymus or thyroid. Malignancies of blood cells, bone marrow cells, B-lymphocytes, T-lymphocytes, lymphocytic progenitors or myeloid cell progenitors may also be treated.

The tumour may be a solid tumour or a non-solid tumour and may be a primary tumour or a disseminated metastatic (secondary) tumour. Non-solid tumours include myeloma; leukaemia (acute or chronic, lymphocytic or myelocytic) such as acute myeloblastic, acute promyelocytic, acute myelomonocytic, acute monocytic, erythroleukaemia; and lymphomas such as Hodgkin's, non-Hodgkin's and Burkitt's. Solid tumours include carcinoma, colon carcinoma, small cell lung carcinoma, non-small cell lung carcinoma, adenocarcinoma, melanoma, basal or squamous cell carcinoma, mesothelioma, adenocarcinoma, neuroblastoma, glioma, astrocytoma, medulloblastoma, retinoblastoma, sarcoma, osteosarcoma, rhabdomyosarcoma, fibrosarcoma, osteogenic sarcoma, hepatoma, and seminoma.

The tumour may be one which presents intracellular or membrane-bound antigens including tumour-specific antigens (for example virally encoded antigens, neo-antigens such as MUC1, antibody idiotypes); antigens which are overexpressed on the surface of tumour cells; oncofoetal antigens including cancer-testis (CT) antigens; or differentiation-antigens (such as tyrosinase and melanocyte antigens). The patient may have an ongoing immune response, such as a Th1 or Th2-type immune response, to antigens on the tumour and may have detectable cytotoxic T cell (CTL) activity, NK cell activity and/or antibody responses against the tumour as determined by, for example, *in vitro* assays.

J. Administration

APCs/T cells/tumour cells prepared by the methods of the present invention for use in immunotherapy are typically formulated for administration to patients with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for

parenteral, intramuscular, intravenous, intra-peritoneal, injection, intranasal inhalation, lung inhalation, intradermal, intra-articular, intrathecal, or via the alimentary tract (for example, via the Peyers patches).

Cells produced by the methods of the invention are typically administered to the patient by intramuscular, intraperitoneal or intravenous injection, or by direct injection into the lymph nodes of the patient, preferably by direct injection into the lymph nodes. Typically from 10^4 to 10^8 treated cells, preferably from 10^5 to 10^7 cells, more preferably about 10^6 cells are administered to the patient.

The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and dosage for any particular patient depending on, for example, the age, weight and condition of the patient. Preferably the pharmaceutical compositions are in unit dosage form. The present invention includes both human and veterinary applications.

The present invention will now be described by way of examples which are intended to be illustrative only and non-limiting, and with reference to the accompanying drawings in which:

Figure 1 shows expression of Notch receptors and Notch ligands in lung tumours; and

Figure 2 shows a graph illustrating co-culture of human CD4+ T cells (HA1.7) with the SCLC line (GLC-19) renders them unresponsive to antigenic restimulation.

EXAMPLES

Example 1 - Small cell lung tumour cells express components of the Notch signalling pathway

Small cell lung tumour cell (SCLC) lines (GLC-19, NCI-H69, NCI-H345, NCI-H711, Lu-165, COR-L24 and COR-L188) and non-small cell lung tumour cell (NSCLC) lines (NCI-H460, MOR/P, COR-L23 and A549) were grown in culture medium (RPMI-1640) supplemented with 10% foetal calf serum in tissue culture flasks and maintained at a density of 2 - 5 x 10^6 cells per ml. Cells were collected and centrifuged at 1500 rpm resuspended in lysis buffer and total RNA extracted. RNA is prepared from cell pellets by homogenisation in guanidium thiocyanate solution followed by CsC1 density centrifugation.

RT-PCR was performed using an Access RT-PCR kit (Promega). One μg of total RNA was used in each reaction together with specific oligonucleotide primers (50 pmol) for the following human genes - Delta1, Jagged (Serrate) 1 and 2, hASH-1, Notch 1, 2 and 3, Radical fringe (controls actin and GAPDH) - and murine genes Hes-1 and Hes-5 under conditions according to the manufacturer's instructions. PCR was performed using a Hybaid machine, dynazyme II polymerase, 1.5 mM Mg^{2+}, 28-35 cycles at an annealing temperature of 60°C.

The sequences of the primers are as follows:

Delta (accession number AF003522)

forward primer	5'-TTTTCTGCAACCAGGACCTGAAC-3'
reverse primer	5'-CACACACTTGGCACCATTAGAAC-3'

Jagged1 (Serrate1) (accession number U73936):

forward primer	5'-TGACAAATATCAGTGTTCCTGCCC-3'
reverse primer	5'-AGCGATAACCATTAACCAAATCCC-3'

Jagged 2 (Serrate2) (accession number AF029778):

forward primer	5'-TGGGACTGGGACAACGATAC-3'
reverse primer	5'-GCAAATTACACCCTTGTTTACACA-3'

Hes-1 (accession number mouse D16464):

forward primer	5'-AATGGAGAATTCCTCCTCCC-3'
reverse primer	5'-TCACCTCGTTCATGCACTCG-3'

Hes-5 (accession number mouse D32132):

forward primer	5'-AAGGAGAAAAACCGACTG-3'
reverse printer	5'-TGTGTTTCAGGTAGCTGAC-3'

hASH-1 (accession number L08424):

forward primer	5'-AACAAGAAGATGAGTAAGGTGGAG-3'
reverse primer	5'-TGGAGTTCAAGTCGTTGGAGTAG-3'

Notch 1 (accession number M73980):

forward primer	5'-GCCAGAACTGTGAGGAAAATATCG-3'
reverse primer	5'-CAGATGGCCTTGCCATTGAC-3'

Notch 2 (accession number X80115):

forward primer	5'-TAACTCCTTCTCTTGCTTGTGCC-3'
reverse primer	5'-ACACACTCGCATCTGTATCCACC-3'

Notch 3 (accession number NM-000435):

forward primer:	5'-TAGGAGGGAGAAGCCAAGTC-3'
reverse primer	5'-AAAAAGGCAATAGGCCCCAG-3'

Radical fringe (accession number AF108139):

forward primer	5'-ATGACAATTATGTGAACGCAAGGA-3'
reverse primer	5'-ACCAGTAGCAAACCAGAACTTGAC-3'

PCR samples were analysed by gel electrophoresis.

The results (Figure 1) demonstrate that SCLC express transcripts for:

Delta1 except for NCI-H345
Jagged1 (Serrate1) except for NCI-H345
Jagged2 (Serrate2) except for GLC-19, NCI-H69, NCI-H345 and NCI-H711
Hes-1
Hes-5 except for GLC 19
hASH-1
Notch2, Notch3 and Radical fringe

All are negative for Notch1

Expression of transcripts for these components of the Notch signalling pathway was also analysed for NSCLC lines (Figure 1) and most notably none of them expressed Delta1.

Example 2 - Induction of unresponsiveness in human T cells following interaction with SCLC cells

Cloned human $CD4^+$ T cells (HA1.7), as described in Lamb et al. (1983), were cultured at 2 x 10^6 cells per well in 12 well tissue culture plates together with irradiated (6000 rads) SCLC (GLC19) at 0.5 x 10^6 per well for 24 hours or alone in tissue culture medium (RPMI-1640 supplemented with 10% human A^+ serum).

The T cells were then restimulated with irradiated (6000 rads) autologous EBV transformed B cells (2.5 x 10^4 per well, as a source of antigen presenting cells) together with increasing concentrations of cognate peptide (influenza) virus haemagglutinin residues 306-318, at 0.1 or 1 μg/ml) or in Interleukin 2 (IL-2;

10% v/v) alone in 96 well tissue culture plates. The cell cultures were pulsed with tritiated thymidine (3[H]-TdR, 0.1 μCi per well) and harvested 72 hours after the initiation of the cultures. The incorporation of 3[H]-TdR as a measure of T cell proliferation was determined by liquid scintillation chromatography.

The T cells (HA1.7) failed to respond to antigen restimulation but were able to proliferate in the presence of exogenous IL-2 demonstrating that the inhibitory effects of the SCLC was not due to cytolytic activity (Figure 2).

The results indicate that the interaction between human $CD4^+$ T cells and SCLC can render the T cells unresponsive to restimulation with specific antigen.

Example 3 – The Serrate-1 gene is expressed in melanoma cell lines

Mouse melanoma cell lines MC57 and B16F10 were cultured in RPMI-1640 medium with 10% FCS at 37°C. Cells were collected and centrifuged at 1500 rpm, resuspended in lysis buffer and total RNA extracted.

RT-PCR was performed using an Access RT-PCR kit (Promega). 50 ng RNA was used in each reaction together with Serrate-1 gene specific oligonucleotide primers (50 pmol) under conditions according to the manufacturer's instructions (T_m for the Serrate oligonucleotides = 58°C).

The sequence of the "forward" Serrate-1 primer is:
5'-GGCTGGGAAGGAACAACCTG-3'
The Serrate-1 "reverse" primer is:
5'-GGTAGCCATTGATCTCATCCAC-3'
Primers specific for Delta are: 5'-GATTCTCCTGATGACCTCGC-3'
and 5'-GTGTTC GTCACACACGAAGC-3'

A PCR-product of the predicted size (330 bp) was observed from RNA obtained from each of the two melanoma cell lines. No Delta-specific PCR product was detected.

These results show that melanoma cell lines express Serrate-1 but not detectable levels of Delta RNA.

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CLAIMS

1. A method for enhancing the reactivity of a T cell toward a tumour cell which method comprises:

(a) isolating a T cell from a patient having said tumour cell present in their body;

(b) exposing the T cell to an agent, which agent is capable of reducing or preventing expression or interaction of an endogenous Notch or Notch ligand in the T cell; and

(c) re-introducing the T-cell into the patient;

wherein the T cell comprises a T cell receptor specific for a tumour antigen expressed by the tumour cell.

2. A method for enhancing the reactivity of a T cell toward a tumour cell which method comprises:

(a) isolating an antigen presenting cell (APC) from a tumour present in the body of a patient;

(b) exposing the APC to an agent, which agent is capable of reducing or preventing expression or interaction of an endogenous Notch or Notch ligand in the T cell when the T cell is contacted with the APC; and

(c) re-introducing the APC into the patient.

3. A method for enhancing the reactivity of a T cell toward a tumour cell which method comprises:

(a) isolating a tumour cell from a tumour present in the body of a patient;

(b) exposing the tumour cell to an agent, which is capable of reducing or preventing expression or interaction of an endogenous Notch or Notch ligand in the T cell when the T cell is contacted with the tumour cell; and

(c) re-introducing the tumour cell into the patient.

4. A method according to any preceding claim wherein the T cell is a tumour

infiltrating lympocyte.

5. A method according to any preceding claim wherein the agent is a nucleic acid sequence which is introduced into the T cell APC or tumour cell.

6. A method according to any preceding claim wherein the agent is a polypeptide or a nucleic acid sequence which encodes a polypeptide selected from a Toll-like receptor, a cytokine a bone morphogenetic protein (BMP), a BMP receptor or an activin.

7. A method according to any one of the preceding claims wherein the endogenous Notch ligand is selected from Serrate, Delta and homologues thereof.

8. A method according to any one of the preceding claims wherein the tumour cell or tumour is a melanoma cell or melanoma, respectively, or a small cell lung tumour cell or small cell lung tumour, respectively.

9. A method according to claim 2 and claims dependent thereon wherein the APC is a dendritic cell.

10. A method of vaccinating a patient against a tumour which method comprises:
(a) administering a tumour antigen expressed by the tumour to the skin of the patient; and
(b) exposing the APC present in the skin of the patient to an agent wherein the agent is capable of reducing or interaction expression or processing of Notch or a Notch ligand in a T cell.

11. A transgenic animal or cell line capable of expressing Notch, Notch ligand or an inhibitor or enhancer of Notch-Notch ligand interactions.

FIGURE 1

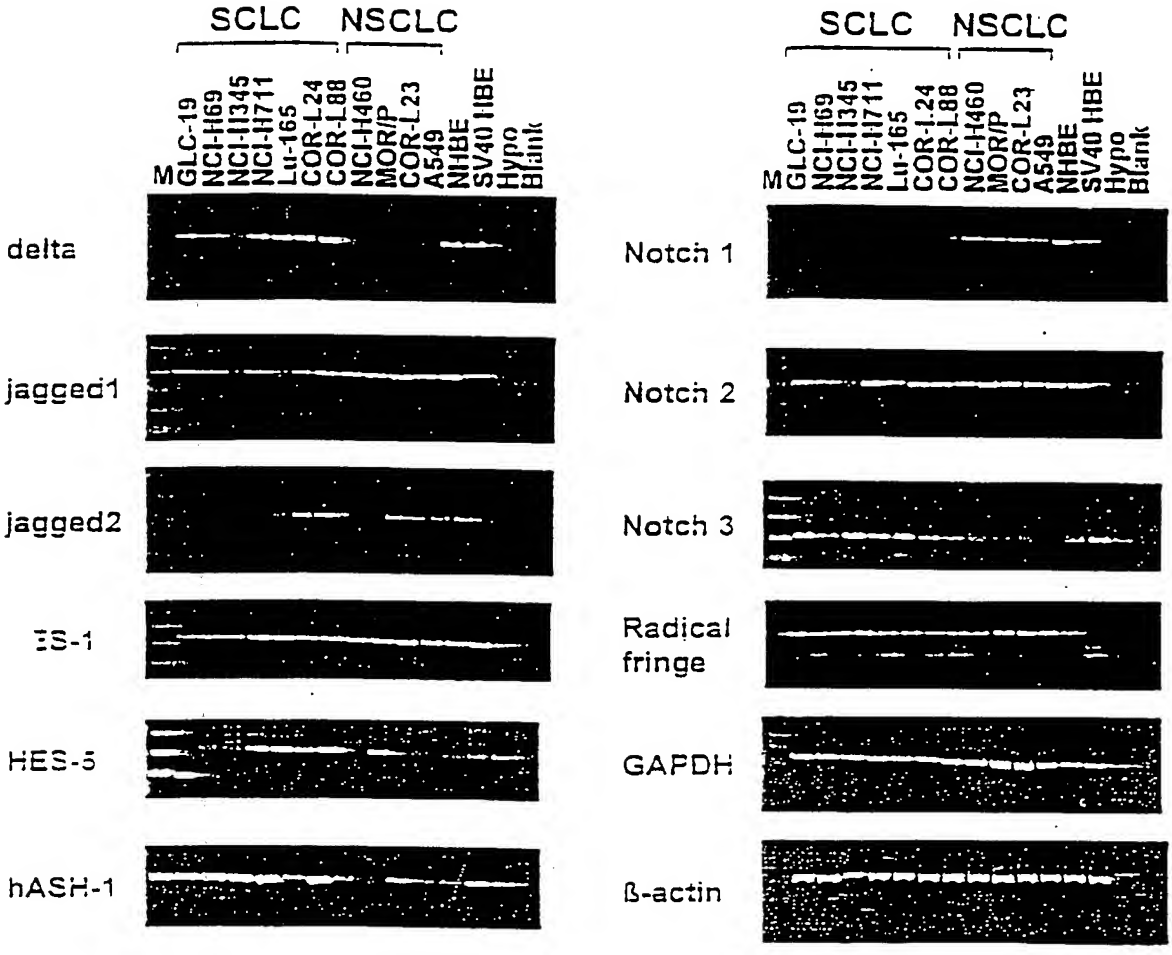
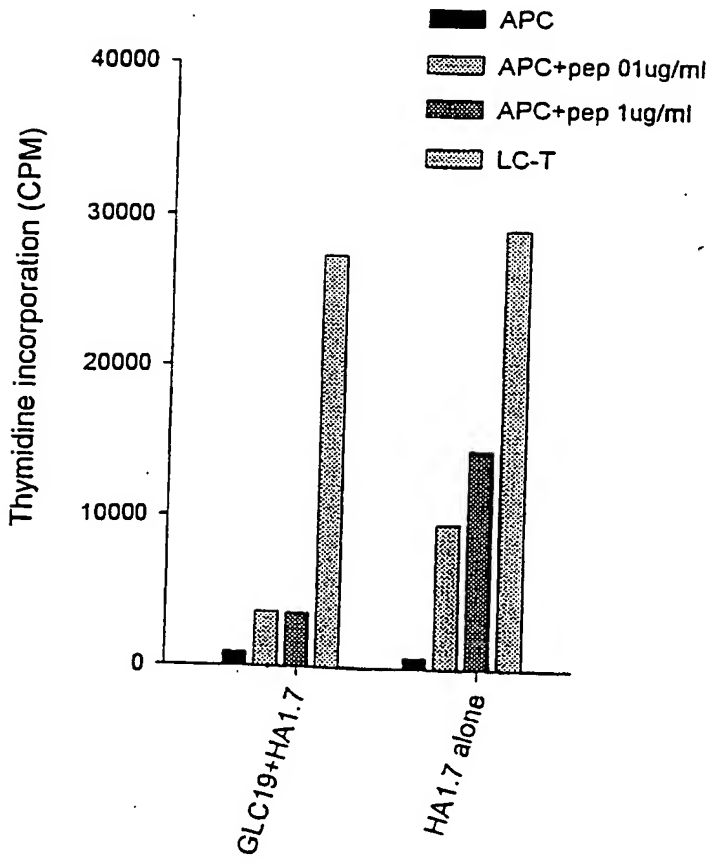

SCLC
NSCLC
M
GLC-19
NCI-H69
NCI-H345
NCI-H711
Lu-165
COR-L24
COR-L88
NCI-H460
MOR/P
COR-L23
A549
NHBE
SV40 HBE
Hypo
Blank
delta
jagged1
jagged2
HES-1
hASH-1
Notch 1
Notch 2
Notch 3
Radical fringe
GAPDH
ß-actin

FIGURE 2


APC
APC+pep 01ug/ml
APC+pep 1ug/ml
LC-T
Thymidine incorporation (CPM)
40000
30000
20000
10000
0
GLC19+HA1.7
HA1.7 alone